Synthesis of 1,25-Dihydroxyvitamin D₃ by Human Endothelial Cells Is Regulated by Inflammatory Cytokines: A Novel Autocrine Determinant of Vascular Cell Adhesion

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Abstract. In addition to its calcitropic function, the secosteroid 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) has potent nonclassical effects. In particular, local production of 1,25D₃ catalyzed by the enzyme 1α-hydroxylase (1α-OHase) may act as an autocrine/paracrine immunomodulatory mechanism. To investigate the significance of this in vascular tissue the expression and function of 1α-OHase in human endothelial cells was characterized. Immunohistochemical and in situ hybridization analyses show, for the first time, the presence of 1α-OHase mRNA and protein in endothelial cells from human renal arteries as well as postcapillary venules from lymphoid tissue. Reverse transcription–PCR and Western blot analyses confirmed the presence of 1α-OHase in primary cultures of human umbilical vein endothelial cells (HUVEC). Enzyme activity in HUVEC (318 ± 56 fmoles 1,25(OH)₂D₃/hr/mg protein) increased after treatment with tumor necrosis factor–α (1054 ± 166, P < 0.01), lipopolysaccharide (1381 ± 88, P < 0.01), or forskolin (554 ± 56, P < 0.05). Functional studies showed that exogenously added 1,25(OH)₂D₃ or its precursor, 25-hydroxyvitamin D₃ (25(OH)D₃), significantly decreased HUVEC proliferation after 72 h of treatment (33% and 11%, respectively). In addition, after 24 h treatment, both 1,25(OH)₂D₃ and 25(OH)D₃ increased the adhesion of monocytic U937 cells to HUVEC (159% and 153%, respectively). These data indicate that human endothelia are able to produce active vitamin D. The rapid induction of endothelial 1α-OHase activity by inflammatory cytokines suggests a novel autocrine/paracrine role for the enzyme, possibly as a modulator of endothelial cell adhesion.

Synthesis of the active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), from its precursor, 25-hydroxyvitamin D₃ (25(OH)D₃), is a pivotal feature of calcium homeostasis. This endocrine process is catalyzed by the enzyme, 25(OH)D₃–1α-hydroxylase (1α-OHase), the expression and regulation of which is best described in the kidney (1). However, production of 1,25(OH)₂D₃ has also been demonstrated at key peripheral sites, including the skin and immune system (2–4). In particular, the expression of the enzyme, 1α-hydroxylase (1α-OHase), may be an important autocrine/paracrine mechanism in peripheral tissues (7).

Relatively little is known about the impact of vitamin D on the cardiovascular system. Increased circulating concentrations of 1,25(OH)₂D₃ reduce the risk of coronary calcification of atherosclerotic vessels (9), and low 1,25(OH)₂D₃ serum levels in patients with renal failure are associated with vascular calcification (10,11). The precise mechanism for this remains unclear and may involve direct effects of 1,25(OH)₂D₃ on calcium accumulation within blood vessels. However, atherosclerosis followed by calcification is known to be a chronic inflammatory process that involves macrophage infiltration (12). The migration of these and other immune cells requires interaction with endothelial cells as part of a highly regulated process involving a range of adhesion molecules (13). Studies elsewhere that have used bovine endothelial cells have demonstrated the presence of intracellular vitamin D receptors (VDR) (14). More recent data have highlighted a possible role for 1,25(OH)₂D₃ as a modulator of cell adhesion (15) and angiogenesis (16). Herein we show for the first time the expression of mRNA and protein for 1α-OHase in human endothelia. Studies in vitro have indicated that synthesis of 1,25(OH)₂D₃ by endothelial cells is stimulated by inflammatory cytokines and may act as a novel autocrine modulator of leukocyte adhesion.

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Materials and Methods

**Tissue Collection and Preparation**

Tissues were obtained in accordance with approval from the local hospital ethics committee. A tissue specimen database from the University of Birmingham was screened for routine surgical kidney and tonsil samples that were classified as surplus to pathologic requirements. For each tissue five different paraffin-embedded samples (n = 5) were used to produce normal kidney and tuberculosis-infected tonsil tissue sections (5 μm thick) on charged slides (Superfrost Plus, BDH, Poole, UK).

**In Situ Hybridization**

In situ hybridization analysis of 1α-OHase mRNA expression was carried out by use of methods described elsewhere (1). Briefly, a cDNA for human 1α-OHase was generated by reverse transcription–PCR by use of mRNA from a human proximal tubule cell line, Digoxigenin (DIG)–labeled antisense and sense cRNA probes were synthesized by use of T7 or SP6 RNA polymerases, after linearization with NorI or Acc65I (DIG RNA labeling kit SP6/T7; Boehringer Mannheim, Sussex, UK). In RNase-free conditions, tissue sections were preheated for 90 min, then dewaxed and permeabilized by use of 0.1% Triton X-100. Tissue sections were then fixed for 10 min at 25 °C. After being rinsed in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, and 10 mM Na2HPO4), sections were refixed at 4 °C for 20 min with 0.4% paraformaldehyde in PBS. Hybridization with antisense DIG-labeled cRNA probes (20 ng/100 μl) was then carried out at 55 °C for 16 h in hybridization buffer (2.5× SSC) that contained 40% (vol/vol) deionized formamide and 12.5% dextran sulfate (wt/wt). The sections were rinsed in water and washed for 10 min at 25°C in 2× SSC, for 20 min at 50°C in 0.1× SSC and for 60 min at 50°C in 0.05× SSC:50% (vol/vol) deionized formamide. Hybridized DIG-labeled probes were detected by use of anti-DIG-alkaline phosphatase Fab fragments (750 U/ml) (Boehringer Mannheim) diluted 1:100 in 50 mM Tris-HCl, at 37°C. After being rinsed in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, and 10 mM Na2HPO4), sections were refixed at 4 °C for 20 min with 0.4% paraformaldehyde in PBS. Hybridization with antisense DIG-labeled cRNA probes (20 ng/100 μl) was then carried out at 55 °C for 16 h in hybridization buffer (2.5× SSC) that contained 40% (vol/vol) deionized formamide and 12.5% dextran sulfate (wt/wt). The sections were rinsed in water and washed for 10 min at 25°C in 2× SSC, for 20 min at 50°C in 0.1× SSC and for 60 min at 50°C in 0.05× SSC:50% (vol/vol) deionized formamide. Hybridized DIG-labeled probes were detected by use of anti-DIG-alkaline phosphatase Fab fragments (750 U/ml) (Boehringer Mannheim) diluted 1:100 in 50 mM Tris-HCl. Visualization of probe was performed by use of 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim) diluted 1:100 in 50 mM Tris-HCl. After being rinsed in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, and 10 mM Na2HPO4), sections were refixed at 4 °C for 20 min with 0.4% paraformaldehyde in PBS. Hybridization with antisense DIG-labeled cRNA probes (20 ng/100 μl) was then carried out at 55 °C for 16 h in hybridization buffer (2.5× SSC) that contained 40% (vol/vol) deionized formamide and 12.5% dextran sulfate (wt/wt). The sections were rinsed in water and washed for 10 min at 25°C in 2× SSC, for 20 min at 50°C in 0.1× SSC and for 60 min at 50°C in 0.05× SSC:50% (vol/vol) deionized formamide. Hybridized DIG-labeled probes were detected by use of anti-DIG-alkaline phosphatase Fab fragments (750 U/ml) (Boehringer Mannheim) diluted 1:100 in 50 mM Tris-HCl. Visualization of probe was performed by use of 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim). Control experiments used the sense cRNA probe and antisense DIG-labeled cRNA probes in the presence of 60-fold excess unlabeled antisense cRNA.

**Immunohistochemistry**

Immunohistochemical analysis of 1α-OHase protein expression was carried out by methods described elsewhere (1). Briefly, paraffin-embedded sections were processed in 0.01 M sodium citrate buffer (pH 6.0) in a pressure cooker at 103 kPa for 2 min. Slides were then incubated with 3% methanol–hydrogen peroxide to block endogenous peroxidase activity and then washed in Tris-buffered saline (pH 7.6). Sections were then incubated with 1α-OHase antisera (1:200) (The Binding Site, Birmingham, UK) in 10% normal swine serum for 45 min at 25°C. After being rinsed with Tris-buffered saline for 15 min, donkey anti-sheep IgG peroxidase conjugate (1:100) was added to sections for 45 min. The localization of protein was visualized by use of precipitating substrate, 3,3’-diaminobenzidine (2.5 mg/ml) (Sigma Chemical Co., Poole, UK), followed by counterstaining with Mayer’s hematoxylin. Control sections included omission of primary antibody and use of primary antibody preabsorbed with a 100-fold excess of immunizing peptide.

**Endothelial Cell Culture**

Primary cultures of human umbilical vein endothelial cells (HUVEC) were prepared as described elsewhere (17). Briefly, the cell pellet obtained from the umbilical vein by collagenase type I digestion (1 mg/ml) was seeded into 1% gelatin–precoated 75 cm2 flasks and initially grown in M199 (Life Technologies-BRL, Paisley, Scotland) with 20% fetal calf serum supplemented with 10 IU/ml heparin, 2 mM L-glutamine, 2 μg/ml endothelial growth factor, 200 IU/ml penicillin, and 200 μg/ml streptomycin (Sigma Chemical Co.). For subculture, confluent cells were harvested with 0.01% ethylenediaminetetraacetic acid–0.1% trypsin. For the experiments, HUVEC were cultured for 48 h by use of plasticware coated with 1% gelatin in Dulbecco’s modified Eagle’s medium:Ham’s F12 (DMEM:Hams F12; Life Technologies-BRL) with 20% charcoal-stripped fetal calf serum with the same supplements mentioned above. HUVEC cells for the experiments were used between passage 1 and 4. For Western blot analysis, 1 × 105 cells were seeded into 75 cm2 flasks; for the RNA extraction, 2.5 × 104 cells into 25 cm2 flasks; for the activity study, adhesion assay, and proliferation study, 1.6 × 104 cells into 24-well plates; and for the intercellular adhesion molecule–1 (ICAM-1)/vascular cell adhesion molecule–1 (VCAM) assay, 0.6 to 1.0 × 105 cells into 96-well plates. HUVEC culture treatment included forskolin (10 μM), tumor necrosis factor–α (TNF-α) (0.5 to 30 ng/ml), 1,25(OH)2D3 (10−7 M), lipopolysaccharide (1 μg/ml), or interferon-γ (300 U/ml). The identity of the endothelial cells was confirmed by positive staining for factor VIII antigen.

**Analysis of 1α-OHase mRNA Expression in HUVEC**

Reverse transcription–PCR analysis of 1α-OHase mRNA expression in HUVEC was carried out by use of methods described elsewhere (18). After reverse transcription of total RNA, PCR amplification was carried out by use of the following primers: 5’-ACGCTGTGGACATGGC-3’ and 5’-GTCAGAGATG- GACAG-CATAT-3’. PCR reactions (20 μl) were set up with use of 1× (final concentration) PCR buffer that contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.5 μM of each primer, and 1 U of Taq DNA polymerase (Promega). Amplification of samples was performed with the use of an initial denaturation step of 95°C for 4 min followed by 35 cycles of 95°C (1 min), 60°C (1.5 min), and 72°C (2 min). A final elongation step of 72°C for 7 min was also included. Purified PCR products were sequenced by direct chain termination sequencing on an automatic DNA sequencer (ABI, Warrington, UK).

**Western Blot Analysis of 1α-OHase Expression in HUVEC**

Further characterization of 1α-OHase protein expression was carried out by Western blot analysis that used HUVEC lysates and the same antisera as that in the immunohistochemical analyses. As a positive control, lysates were also prepared from the human proximal tubule cell line HKC-8 cells, as described elsewhere (18). Tissue samples and cell preparations were washed in PBS that contained 0.5 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) and homogenized by use of a glass Teflon homogenizer. Cell membranes were pelleted at 2900 × g at 4°C for 5 min and the supernatant, which contained the 1α-OHase protein, was stored at −80°C. Aliquots of protein were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (5.5 μg per lane) and electroblotted onto Immobilon P membrane, as described elsewhere (18). Filters were blocked and incubated with primary antibody at 1:500 dilution, and secondary antibody (horseradish peroxidase–conjugated; Amersham, Aylesbury, UK) at 1:75,000 dilution. Protein for 1α-OHase was detected by enhanced chemiluminescence (Amersham) after exposure of filters to x-ray film for 10 to 30 s. Control experiments were included where primary antibody was omitted or primary antibody
was preabsorbed with a 100-fold excess of immunizing peptide. No protein bands were detected in these controls (data not shown).

**Measurement of 1,25(OH)₂D₃ Production by HUVEC**

Vitamin D metabolism in subconfluent HUVEC (n = 3) was assessed by use of thin-layer chromatography methods described elsewhere (18). Briefly, cells were seeded in 24-well plates and grown to 50% confluence before transfer to DMEM:HAM F12 (Life Technologies-BRL) with 20% charcoal-stripped fetal calf serum supplemented as described above for 48 h, with treatment for the last 24 h. Cells were then incubated in 400 μl DMEM:HAM F12 with 10 nM [³H]-25(OH)D₃ (specific activity 180 Ci/mmmol, Amersham) for 4 h at 37°C. The reaction was terminated by freezing at −20°C. Cell extracts and medium were combined, and vitamin D metabolites were extracted in 2.5 ml of chloroform:methanol (4:1 vol/vol). After evaporation of the organic phase, steroids were resuspended in 50 μl of dichloromethane and separated on silica TLC plates in dichloromethane:isopropanol (9:1 vol/vol). Standard lanes were included that contained only [³H]-25(OH)D₃ or [³H]-1,25(OH)₂D₃. Production of [³H]-1,25(OH)₂D₃ was measured on a Bioscan System 200 imaging TLC plate scanner (Bioscan Inc., Edmonds, WA). Residual cell monolayers from parallel wells were lysed and assayed for total cellular protein by use of a Bio-Rad protein assay (Bio-Rad, Melville, NY). Data were reported as fmols 1,25(OH)₂D₃ produced/h per mg cellular protein.

**Analysis of the Effect of Vitamin D Metabolites on HUVEC Proliferation**

After specific treatments, cells were incubated with 0.5 μCi ³H-thymidine (specific activity 80 Ci/mmol; Amersham) for the last 6 h of culture incubation. Unlabeled thymidine was added for the last 5 min to displace any nonspecific uptake of ³H-thymidine. Cells were then washed twice in PBS, followed by 1 ml of cold 5% TCA to precipitate proteins, and left on ice for 20 min. The liquid layer was then removed and drained. An aliquot (250 μl) of 0.1 M sodium hydroxide was added to the cells and left at room temperature for 30 min on a shaker. The resulting solubilized nuclear material was then transferred to 4 ml of scintillant and radioactive counts determined by scintillation counting. Data were reported as mean ± SD of radioactivity determinations per minute (n = 4).

**Analysis of the Effect of Vitamin D Metabolites on Endothelial Cell–Monocyte Adhesion**

The functional impact of exogenous added and endogenously produced 1,25(OH)₂D₃ on HUVEC was assessed by analysis of changes in endothelial cell-monocyte adhesion. Binding of untreated proliferating myelomonocytic U937 cells to HUVEC (n = 4) was determined by use of a colorimetric method, as described elsewhere (19). Briefly, medium was removed from confluent endothelial cell layers after treatment for 24 h. A suspension of 1 × 10⁶ unstimulated U937 cells/500 μl DMEM:HAM F12/well was added and coculture incubated for 15 min under continuous rotational movement at 37°C. The nonadhering monocytes were removed by washing three times with sterile PBS, and those adhering to the endothelial cells were fixed for 20 min with 1.0% glutaraldehyde. After washing and air drying, cells were stained with a crystal violet solution (0.1% wt/vol) for 5 min, rinsed three times with sterile PBS, and solubilized in Triton X-100 (1.0%) overnight. Absorbance readings for the solubilized U937 cells were measured at 595 nm by use of a spectrophotometer (Titrerox Multiscan; Labsystem Ltd., Basingstoke, UK). The results were subtracted from background readings resulting from staining of endothelial cells with the corresponding treatment alone (U937 cells absent).

The corrected readings were then taken to be proportional to the number of monocytes bound and were expressed as percentage adherence of control (100% representing untreated endothelial cell adhesion of U937). Expression of ICAM-1 and VCAM-1 by HUVEC was assessed by use of enzyme-linked immunosorbent assay protocols described elsewhere (19).

**Statistical Analyses**

Assays for 1α-1Hase (n = 3), the monocyte adhesion (n = 4), and the endothelial cell proliferation assays (n = 4) are reported as the mean ± SD of at least three experiments with the use of different umbilical cords. Statistical analyses were performed by use of one-way ANOVA linked to Turkey-Kramer multiple comparison posttests (Instat version 2.04a computer program, GraphPad Software, Inc., San Diego, CA).

**Results**

Analysis of normal human renal tissue sections by in situ hybridization showed 1α-1Hase mRNA expression in endothelial cells of large arteries and veins (Figure 1A) as well as small arteries and veins (data not shown). No expression was detectable in the other parts of the blood vessel wall. Control analyses that used sense cRNA probes (data not shown) or coinoculation of labeled probe with a 60-fold excess of unlabeled antisense 1α-1Hase cRNA (Figure 1C) showed no staining. Parallel immunohistochemical analyses showed similar results, with 1α-1Hase protein detectable in endothelial cells of renal large arteries (Figure 1B). No staining was observed in renal arteries when antisera was preabsorbed with immunizing peptide (Figure 1D).

Protein for 1α-1Hase was also detectable in blood vessels of lymphatic tissue, particularly in tissue affected by chronic inflammation. As shown in Figure 2A, strong expression of 1α-1Hase protein was observed in the cuboidal endothelia of post capillary venules of a tuberculosis-affected tonsil. Staining was also detected in sinusoidal endothelia cells from spleen (data not shown). Parallel analysis of sequential tissue sections indicated that cells expressing 1α-1Hase were positive for CD34 (endothelium) (Figure 2B) and negative for actin (smooth muscle) (Figure 2C) and CD68 (monocytes) (Figure 2D).

Studies in vitro that used HUVEC confirmed the identity of 1α-1Hase in endothelial cells and also highlighted a novel function for locally produced 1,25(OH)₂D₃. A reverse transcription–PCR product (542 bp) (data not shown) and a single Western blot protein species (56 kD) that corresponded to the reported renal 1α-1Hase (Figure 3A) were detectable in control HUVEC. Densitometric analysis of Western blots (data not shown) showed a 50% increase in 1α-1Hase expression after 24 h treatment with forskolin, whereas all the other treatments were without effect. Analysis of ³H-25(OH)D₃ metabolism confirmed the capacity for 1α-hydroxylation in HUVEC (Figure 3B). However, in contrast to protein analyses, synthesis of 1,25(OH)₂D₃ increased after 24 h of treatment with forskolin (10 μM), TNF-α (5 ng/ml), or lipopolysaccharide (1 μg/ml). Interferon-γ (300 U/ml) did not increase, and 1,25(OH)₂D₃ itself did not suppress 1α-1Hase activity significantly.

Further studies were carried out to assess the functional significance of local conversion of 25(OH)D₃ to 1,25(OH)₂D₃.
in cultured endothelial cells, with respect to a possible autocrine function for 1α-OHase. Studies of 3H-thymidine incorporation showed that 1,25(OH)2 D3 inhibited HUVEC proliferation in a dose-dependent and time-dependent fashion (Figure 4). This response was not as potent as that observed for TNF-α (5 ng/ml). Furthermore, combined treatment with TNF-α and 1,25(OH)2 D3 produced only a modest additive effect. Of interest, the substrate for 1α-OHase, 25(OH)D3, also significantly inhibited HUVEC proliferation, despite its status as an inactive metabolite of vitamin D. However, this effect was much less potent than that observed for 1,25(OH)2 D3, and combination with TNF-α showed no apparent additive inhibition of cell proliferation. Data in Figure 5A show that vitamin D metabolites were also able to increase the capacity for adhesion of myelomonocytic U937 cells to adhere to HUVEC. TNF-α (5 ng/ml), used as a positive control, produced the highest level of U937 adhesion to HUVEC in 24-h treatments. However, both 1,25(OH)2 D3 and 25(OH)D3 significantly enhanced the adhesion of U937 cells after the same incubation period. Analysis of HUVEC ICAM-1 and VCAM-1 expression by enzyme-linked immunosorbent assay (Figure 5B) indicated that expression of both the cell adhesion molecules was up-regulated by 5 ng/ml TNF-α: ICAM-1, 120-fold ± 11 compared with untreated HUVEC and VCAM-1, 17-fold ± 0.5. In contrast 1,25(OH)2 D3 and 25(OH)D3 (1 to 100 nM) were without effect.

**Discussion**

Synthesis of 1,25(OH)2 D3 as a consequence of the peripheral expression of 1α-OHase has been documented at several extrarenal sites. In particular, activated macrophages associated with inflammatory disorders such as sarcoidosis, tuberculosis, and rheumatoid arthritis have a high capacity for 1,25(OH)2 D3 production, which can, in turn, lead to hypercalcemia (2–4,20–22). More recently, the human cDNA for 1α-OHase was cloned from keratinocytes (23), which appear to synthesize 1,25(OH)2 D3 as part of an autocrine/paracrine component of epidermal cell differentiation (24). Although 1α-OHase expression throughout the body appears to be due to a single cDNA species (25), there are important differences in
the regulation of renal and extra-renal 1,25(OH)_{2}D_{3} production. Most notably, peripheral 1α-OHase activity does not appear to be subject to the exquisite autoregulation observed for this enzyme in the kidney. Thus, extrarenal synthesis of 1,25(OH)_{2}D_{3} is very much dependent on the availability of its substrate 25(OH)D_{3}, which is in turn a direct reflection of dietary and environmental access to parental vitamin D. In this way, extrarenal 1α-OHase occupies a unique position at the interface between the environmental and genetic factors that determine the actions of vitamin D.

The presence of mRNA and protein for 1α-OHase in the human vasculature confirms previous in vitro analyses that have used bovine tissue, which showed that endothelial cells are capable of synthesizing 1,25(OH)_{2}D_{3}; a possible autocrine/intracrine mechanism was illustrated by the coexpression of 1α-OHase with VDR and the inhibition of endothelial cell proliferation after treatment with exogenous 1,25(OH)_{2}D_{3} (14). More recent work has confirmed the sensitivity of endothelial cells to 1,25(OH)_{2}D_{3}, which suggests in particular that modulation of angiogenesis may be an important component of the reported anticancer effects of this hormone (16). However, as shown in Figure 1, the presence of mRNA and protein for 1α-OHase in blood vessels from normal tissue suggests that endothelial production of 1,25(OH)_{2}D_{3} is not associated exclusively with tumors. Indeed, subsequent analysis of lymphatic tissue suggests that endothelial expression of 1α-OHase is more likely to be a feature of inflammatory disease. In this respect, vascular synthesis of 1,25(OH)_{2}D_{3} more closely resembles the 1α-OHase activity observed in activated macrophages (20–22). In contrast to its renal counterpart, macrophage/endothelial cell 1α-OHase is poorly induced by cAMP activators such as forskolin and shows no self-regulation after treatment with 1,25(OH)_{2}D_{3} (5). Instead, the most potent inducers of 1,25(OH)_{2}D_{3} in macrophages and endothelial cells appear to be mitogens such as lipopolysaccharide and cytokines including TNF-α and interleukin-1 (2,3,5,7,21,22). The precise mechanism for this remains unclear. In data presented here, only treatment with forskolin showed any correlation between increased synthesis of 1,25(OH)_{2}D_{3} and 1α-OHase protein expression. This suggests a mechanism for inflammation-induced 1α-OHase activity that is distinct from calcitropic transcriptional regulation. For example, studies elsewhere by Adams et al. (26) have shown that macrophage synthesis of 1,25(OH)_{2}D_{3} requires coordinated interaction with nitric oxide. In view of the important role of nitric oxide and its generating enzyme, nitric oxide synthase, in generalized vas-
1,25(OH)2 D3 and coronary calcification is clearly different. The reciprocal relationship between circulating levels of vitamin D in the progression of atherosclerosis, specifically 1,25(OH)2 D3, fulfills a different function to the classical response initiated by the potent calcemic responses observed in other tissues, including renal stone formation. Thus, data presented here suggest that 1α-OHase activity in endothelial cells is more likely to be associated with nonclassical actions of 1,25(OH)2 D3. The most well characterized of these are the potent immunosuppresive and antiproliferative effects of 1,25(OH)2 D3 and its synthetic analogs, which have led to potential treatments for graft rejection, autoimmune disease (5–8), and specific cancers (28–31). Despite this, the precise role of 1,25(OH)2 D3 as a physiologic modulator of noncalcitropic peripheral tissue function is far less clear. We have postulated that the localized production of 1,25(OH)2 D3 by activated macrophages and the modulatory effect of this on adjacent leukocyte populations constitutes a novel cytokine-like function for this hormone (7). In a similar fashion, reports elsewhere that have documented the expression and activity of 1α-OHase in normal human colon and prostate tissue suggest that local synthesis of 1,25(OH)2 D3 by these tissues is a contributing factor in associated tumors (32–35). Specifically, the presence of 1α-OHase activity in primary cultures of normal prostate cells conferred antiproliferative sensitivity to 25(OH)D3 as well as 1,25(OH)2 D3, whereas the loss of the enzyme in advanced prostate tumors correlated with decreased sensitivity to 25(OH)D3 (32). The level of 1,25(OH)2 D3 production by primary HUVEC cultures was significantly lower than that observed with primary prostate tissue (4000 fmoles/h per mg protein versus 400 fmoles/h per mg protein). Nevertheless, treatment with 100 nM 25(OH)D3 for 72 h produced a small but significant decrease in HUVEC proliferation. The fact that 25(OH)D3 has a binding affinity for the VDR that is 1000 lower than that for 1,25(OH)2 D3 suggests that the antiproliferative response to 25(OH)D3 in HUVEC is due to autocrine activation via 1α-OHase. This effect was not enhanced by cotreatment with the 1α-OHase inducer TNF-α. However, at the concentrations and incubations periods used in this study, TNF-α is itself a potent antiproliferative agent, and, as such, coincident local conversion of 25(OH)D3 to 1,25(OH)2 D3 is likely to have a relatively modest effect.

Studies elsewhere by ourselves and others (15,36,37) have shown that 1,25(OH)2 D3 is also a potent modulator of cell adhesion. In addition, leukocyte-endothelial cell adhesion is known to play an important role in both normal vascular function and pathologic conditions such as atherosclerosis. Further studies were therefore carried out to investigate the interaction between these two mechanisms. Treatment with either 25(OH)D3 or 1,25(OH)2 D3 stimulated adhesion of mononuclear U937 cells to HUVEC after only 24 h treatment, which indicates that this was a more sensitive autocrine response than inhibition of cell proliferation. Induction of cell adhesion by 25(OH)D3 and 1,25(OH)2 D3 was not as potent as that observed for TNF-α, a classical stimulator of leukocyte-endothelial cell adhesion. The effects of TNF-α are mediated, in part, via induction of ICAM-1 and VCAM-1 (38), and this was confirmed in our enzyme-linked immunosorbent assay data. In contrast, response to 25(OH)D3 or 1,25(OH)2 D3 appears to be independent of these two adhesion molecules, which suggests a mechanism that is similar to but distinct from that observed for inflammatory cytokines. We have reported elsewhere similar observations for LDL, which stimulated monocyte-mesangial cell adhesion without any significant response than inhibition of cell proliferation. Induction of cell adhesion by 25(OH)D3 and 1,25(OH)2 D3 was not as potent as that observed for TNF-α, a classical stimulator of leukocyte-endothelial cell adhesion. The effects of TNF-α are mediated, in part, via induction of ICAM-1 and VCAM-1 (38), and this was confirmed in our enzyme-linked immunosorbent assay data. In contrast, response to 25(OH)D3 or 1,25(OH)2 D3 appears to be independent of these two adhesion molecules, which suggests a mechanism that is similar to but distinct from that observed for inflammatory cytokines. We have reported elsewhere similar observations for LDL, which stimulated monocyte-mesangial cell adhesion without any significant change in ICAM-1 or VCAM-1 expression (19). A variety of different adhesion molecules may be involved in this process, including αVβ3 integrin, which is known to be expressed by endothelial cells (39) and is transcriptionally regulated by 1,25(OH)2 D3 (36). We can therefore postulate that the induction of vascular-leukocyte adhesion by vitamin D metabolites fulfills a different function to the classical response initiated by inflammatory cytokines. In particular, the effects of vitamin D on coronary calcification suggest that leukocyte adhesion induced by 1,25(OH)2 D3 and possibly paracrine effects of 1,25(OH)2 D3 on transmigrating leukocytes have a more beneficial effect on vascular function.
The data presented in this study identify endothelial cells as an important new site of extrarenal synthesis of 1,25(OH)2D3. In common with other extrarenal tissues, endothelial 1α-hydroxylase appears to fulfil an paracrine/autocrine function; namely, increased endothelial synthesis of 1,25(OH)2D3 may acts at a local level to modulate the effects of inflammatory cytokines on the vasculature by promoting leukocyte adhesion. To date, there have been no specific studies of vascular function or pathology in patients with hereditary 1α-OHase or VDR dysfunction or, indeed, their equivalent knockout mice. Further studies that use these and other model systems will help to further define the role of vascular vitamin D metabolism in the pathogenesis and management of vascular disease.

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